Kidney International, Vol. 44 (1993), pp. 87–90

Intra-peritoneal free elastase in CAPD peritonitis

KIERON L. DONOVAN, STEVE PACHOLOK, JOHN L. HUMES, GERALD A. COLES, and JOHN D. WILLIAMS

Institute of Nephrology, Royal Infirmary, Cardiff, Wales, United Kingdom and Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, USA

Intra-peritoneal free elastase in CAPD peritonitis. Neutrophil (PMN) recruitment into the peritoneum during acute bacterial peritonitis is an important part of the host defense barrier in CAPD patients. However, the subsequent phagocytosis of bacteria may also lead to PMN degranulation and the release of lysosomal enzymes. We determined the concentration of neutrophil elastase, both in complex with its natural inhibitor α1Pi (Eα1Pi), and in uncomplexed, free form, in infected and normal CAPD peritoneal fluid by ELISA. In addition elastase activity was estimated in a casein degradation assay. Infected fluid contained a median (range) of 1.4 nM (0 to 9.2) free elastase by ELISA and 1.2 nM (0 to 11.9) activity. There were strong correlations between the peritoneal leukocyte count and both immunoreactive elastase and activity ($r = 0.816$, $P < 0.001$, 0.687, $P < 0.01$, respectively). In contrast, normal fluid contained 0.0 nM (0 to 0.32) immunoreactive elastase ($P < 0.01$) and 0.0 nM (0 to 0.6) elastase activity ($P < 0.001$). Eα1Pi complexes were raised significantly during peritonitis at 6.2 nM (0 to 34.3) and were barely detectable in normal fluid 0.0 nM (0 to 0.17; $P < 0.005$). The study shows that small but significant quantities of uninhibited elastase can be detected in the peritoneal fluid of CAPD patients with acute bacterial peritonitis. This observation may have important implications for the pathogenesis of peritoneal membrane damage and the phlogistic response to infection.

Peritonitis is a serious and common complication of CAPD which may lead to peritoneal fibrosis and loss of ultrafiltration [1]. The condition is initially characterized by a rapid influx of PMN into the peritoneal fluid. The eradication of invading bacteria by intra-peritoneal phagocytes represents an important facet of the host defense mechanism. The phagocytosis of bacteria by PMN, however, may also result in their activation and subsequent degranulation [2] with the discharge of potentially harmful proteinases such as elastase into the local tissues. The release of such enzymes is balanced by the presence of inhibitors such as alpha 1 proteinase inhibitor (α1Pi). In normal extracellular sites there is usually an excess of α1Pi and its rapid combination with elastase inhibits enzyme activity and limits tissue damage [3]. However, fresh CAPD fluid contains no α1Pi and, even after equilibration, levels would only be expected to reach ~5% of plasma levels [4]. This artificial milieu thus comprises a relatively inhibitor free environment. The present study estimated intraperitoneal PMN elastase in CAPD patients with acute bacterial peritonitis and in normal CAPD fluid by both immunological and functional assays.

Received for publication October 21, 1992
and in revised form February 3, 1993
Accepted for publication February 4,1993

Methods

Patients and sample collection

CAPD fluid samples were collected from patients presenting with acute peritonitis ($N = 15$) and from non-infected patients ($N = 13$) who served as controls. All subjects had been established on CAPD for more than six months. Peritonitis was diagnosed on the basis of cloudy bags associated with a raised peritoneal leukocyte count ± abdominal discomfort. Fluids were collected at first presentation of peritonitis, after dwell times of up to four hours, and cells and debris were removed by centrifugation within 30 minutes of collection. No patient was on antibiotics or heparin at presentation. The resultant supernatants were aliquoted and frozen at −70°C until analysis. A sample was also sent for routine microscopy and microbiological assessment. In addition, samples were obtained from another 19 patients, after a 12-hour intraperitoneal fluid dwell time, for estimation of maximal α1Pi concentration.

Eα1Pi assay

Eα1Pi levels were determined by a double antibody ELISA capable of detecting human neutrophil elastase in complex with α1Pi [5]. Individual uncomplexed components were not recognized. Briefly, microtiter wells were coated with sheep anti-human PMN elastase (ICN Biochemicals, Irvine, California, USA) diluted 1:1000 in Vollers buffer, incubated overnight at 4°C and washed. Plasma or peritoneal fluid samples, diluted in buffer containing 0.1% BSA, were then added to the wells and incubated for one hour. Rabbit anti-α1Pi (Calbiochem, San Diego, California USA) diluted 1:500 in buffer was added and incubated for a further hour at room temperature. Subsequent washes (× 3) were followed by the addition of anti-rabbit IgG conjugated to horse radish peroxidase (Calbiochem) diluted 1:100 in buffer and the plates incubated for 60 minutes at room temperature. Substrate chromophore was added for 15 minutes and the reaction stopped by adding 2 M H_2SO_4. Absorbance was measured at 492 nM on an automated plate reader. The assay was calibrated using known concentrations of Eα1Pi complexes formed by adding excess α1Pi (Athens Research, Georgia, USA) to pure human neutrophil elastase. The detection limit was 0.3 nM.

Table 1. Clinical details of patients presenting with acute bacterial peritonitis

Patient	Leukocytes mm^{-3}	Organism	EαlPi	Elastase	Elastase nM (activity)
			nM (ELISA)		
1	710	Staph aureus	0.0	0.0	ND
2	1260	Staph aureus	3.5	1.9	2.2
3	1330	Staph epi	6.2	2.9	0.9
4	240	Pseudomonas	0.0	0.0	0.0
5	2900	Pseudomonas	6.8	2.2	1.4
6	5300	Staph aureus	19.7	6.4	10.2
7	1640	Nil	0.0	0.4	0.6
8	503	Staph epi	0.0	0.0	0.6
9	1100	E faecalis	7.2	1.3	10.0
10	820	Staph aureus	3.5	0.7	6.0
11	9700	Coliforms	34.3	9.2	11.9
12	2030	Staph aureus	9.5	1.4	0.0
13	1520	Staph epi	22.0	3.1	0.0
14	1680	Staph aureus	1.1	0.3	1.1
15	4965	Staph epi	16.4	0.0	ND

Abbreviations are: Staph epi, *staphylococcus epidermis*; Staph aureus, *staphylococcus aureus*; E faecalis, *Enterococcus faecalis*; ND, not determined.

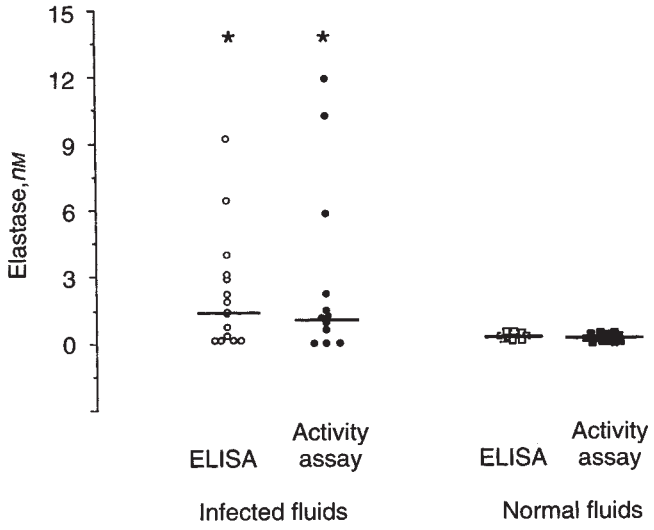

Fig. 1. *Comparison of elastase concentration by trap ELISA (open symbols) and activity (closed symbols) in infected and normal CAPD fluids.* * $P < 0.05$ infected vs. non-infected (Mann Whitney U test), — = median value.

Free elastase ELISA

Elastase unbound to α1Pi was estimated by repeating the Eα1Pi assay following an initial pre-incubation of each sample with an excess of α1Pi (18 μM) for 60 minutes. In this step free elastase unbound to α1Pi is trapped by the exogenous α1Pi. A difference in the measured concentration of Eα1Pi as compared to the original non pre-incubated sample was the estimated free elastase by ELISA.

Elastase activity assay

Elastase proteolytic activity was determined using azo casein substrate modified from Barret [6]. Samples were concentrated tenfold in Centricon 10 tubes (Amicon, USA) at 5000 g. One hundred microliters of concentrated sample were incubated with 125 μl azocasein (6% wt/vol pH 7.5) and 175 μl sample buffer (1.25 M Tris, 2.5 M KCl, pH 7.5) and 75 μl of either PBS or α1Pi (1 mg/ml in PBS). In addition pure neutrophil elastase standards (0.1 to 100 ng) and negative control incubations of PBS, both ± α1Pi, were also included. After 24 hours of incubation at 37°C 1.5 ml of cold 3% trichloracetic acid was added and the mixtures were filtered through Whatman No. 1 paper. Absorbance at 366 nM was measured for each sample and activity was expressed as equivalents of elastase from the standard curve obtained with pure elastase samples and corrected for concentration factor.

Assay for intraperitoneal α1Pi

Concentration of α1Pi in 16-hour dwell PD samples was determined by rate nephelometry using a Beckman Array system (Beckman Instruments Inc., Brea, California USA). This is an established regional assay in our Protein Reference laboratory.

Statistical analysis

All data are expressed in non-parametric form. Unpaired data were compared using the Mann Whitney U test to provide z values, with P derived from the z distribution. Paired data were evaluated using Wilcoxon's signed rank test. For both tests $P < 0.05$ was considered to be significant. Correlations between separate variables were made using linear regression analysis and expressed as both r and P values; $P < 0.05$ was significant.

Results

Clinical details of all infected patients are outlined in Table 1. Infected CAPD fluid contained significantly more free elastase than normal fluid (Fig. 1). Median (range) concentration was 1.4 nM (0 to 9.2) by ELISA ($N = 15$) and 1.2 nM (0 to 11.9) by activity assay ($N = 13$). Peritoneal leukocyte count correlated strongly with both immunoreactive elastase (Fig. 2) and activity ($r = 0.687$, $P = 0.0095$). Proteolytic activity in infected fluids was fully inhibited by 1 mg/ml α1Pi (Fig. 3). There was a significant correlation ($r = 0.694$, $P = 0.0085$) between ELISA and activity data (Fig. 4) in infected fluid. In eight patients Eα1Pi and free elastase were measured both during and six weeks after recovery from peritonitis. Elevated Eα1Pi and free elastase were seen in the infected fluid but were both undetectable after recovery (data not shown). Eα1Pi complexes were significantly raised during infection as compared to normal 6.2 nM (0 to 34.3) versus 0 (0 to 0.17; $P < 0.005$). There were no concomitant changes in either blood PMN count 5.88×10^9/liter (4.10 to 8.70) versus 5.05 (4.61 to 7.20), or plasma Eα1Pi concentration 2.7 nM (1.9 to 4.8) versus 3.1 nM (1.8 to 3.7).

The concentration of α1Pi in equilibrated CAPD fluid was 0.07 mg/ml (<0.03 to 0.12). Reference range in plasma was 1.5 ± 0.5 mg/ml. Median peritoneal/plasma ratio was estimated as 0.05.

Discussion

This present study demonstrates that during acute bacterial peritonitis in CAPD patients, nanomolar quantities of uninhibited elastase can be detected within the peritoneal cavity by both a specific immunoassay and an activity assay. The PMN primary granule enzyme elastase is a powerful serine proteinase

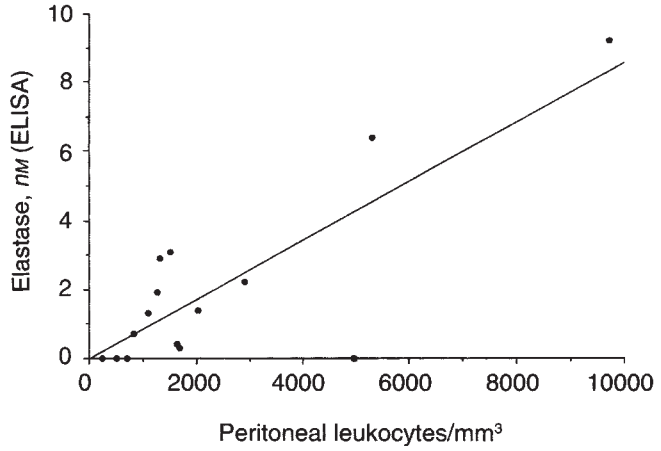

Fig. 2. *Correlation between peritoneal leukocyte count (mm^{-3}) and free elastase measured by ELISA in acute peritonitis samples.*

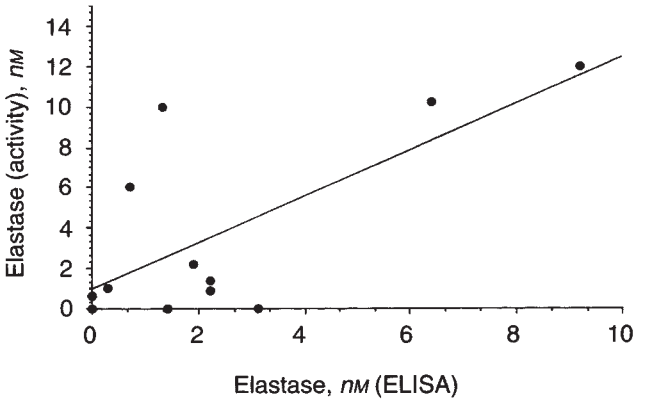

Fig. 4. *Comparison of elastase activity and immunoreactivity in infected CAPD fluid by linear regression analysis. $r = 0.694$, $P = 0.0085$.*

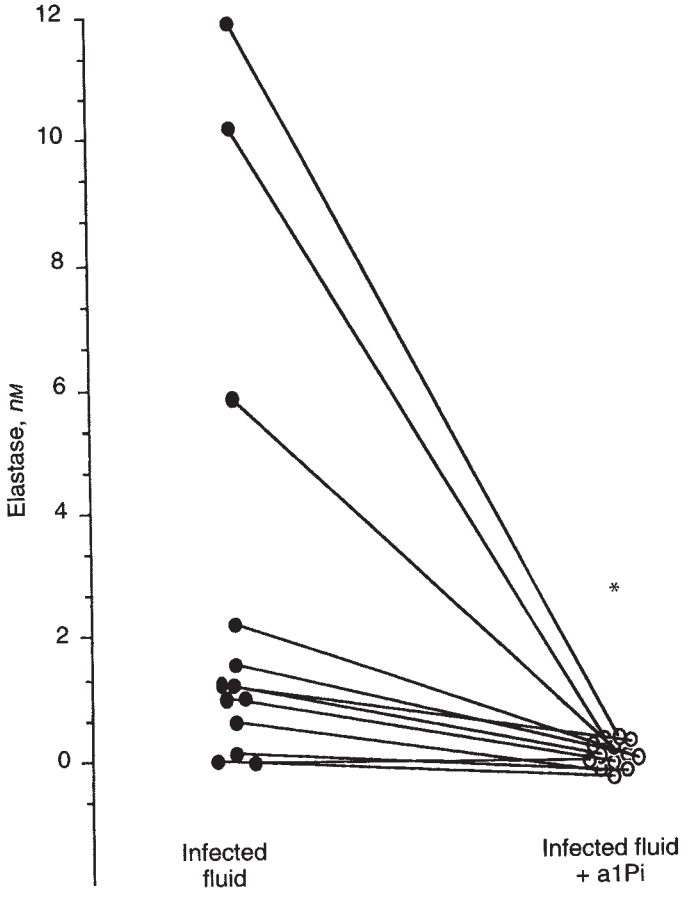

Fig. 3. *Changes in elastase activity of infected PD fluid before and after the addition of excess α1Pi.* * $P < 0.05$ activity without α1Pi vs. with α1Pi (Wilcoxon's signed rank test). $r = 0.816$, $P = 0.0002$.

which can damage both basement membranes [7] and intact cells [8]. Histological studies have shown that considerable damage, including areas of denudation, occurs to the mesothelial cell layer during infection [1] and repeated infection can lead to peritoneal fibrosis, loss of ultrafiltration and, ultimately, technique failure. In addition elastase can cleave plasma proteins such as complement [8] to form chemotactic factors which enhance local phagocyte recruitment.

During the phagocytosis of bacteria by PMN, cell activation and degranulation are known to occur [2]. Under normal circumstances extracellular elastase rapidly complexes with the excess of surrounding inhibitors, of which α1Pi is the most important [9]. The formation of enzymatically-inactive Eα1Pi complexes thus limits local proteolytic tissue damage. However, the peritoneal cavity of CAPD patients comprises an artificial milieu. Fresh CAPD fluid contains no α1Pi, and during the course of a 12-hour dialysis dwell we have shown that it reaches only 5% of plasma concentration.

In the present study Eα1Pi complexes were detected in nM concentration in infected fluid, indicating the presence of elastase and its subsequent binding to α1Pi. The elastase is unlikely to have diffused into the peritoneal cavity from the plasma compartment, since the plasma levels of Eα1Pi were identical in both infected and non-infected patients whereas significant levels of the complex were found only in infected fluids. We believe that the elastase is most likely to have arisen from PMN which invaded the peritoneal cavity during acute bacterial infection. However, although significant correlations between PMN numbers and elastase support this hypothesis, the relatively loose fit of the data to linearity (as evidenced by the r values of <0.83) warrants further comment.

The elastase content of human PMN may vary by up to 300% [8], and its release during degranulation is dependent upon both the type and magnitude of cell stimulus [2]. Our data represent analysis of the intra-peritoneal elastase release from 13 patients' PMN interacting with differing bacterial strains and concentrations. In addition, there is now evidence that peritoneal mesothelial cells can up-regulate the expression of adhesion molecules in response to inflammatory stimuli [10]. This could lead to adherent PMN releasing elastase, further increasing errors in simple comparisons of elastase versus PMN count. Bearing these potential sources of error in mind it is not surprising that loose, albeit significant, fits were seen.

There has been one other report of elevated Eα1Pi complexes in both plasma and peritoneal fluid during peritonitis which showed mean intraperitoneal Eα1Pi levels of ~40 nM [11].

However, our finding of free elastase by both ELISA and functional assay during infection suggests that this normal inhibitory process is inefficient within the peritoneal cavity during peritonitis. Reactive oxygen species released by activated PMN can oxidize critical methionine residues within the elastase binding site of α1Pi [8], and although the inhibitor may still be detectable by immunoassay, its elastase inhibitory capacity may be reduced by up to 1000-fold [8]. A recent study has demonstrated that α1Pi in the fluid of patients with peritonitis is oxidized, and that in this form it is much less efficient at inhibiting elastase mediated degradation of a chromogenic substrate, and TNFα induced release of PAF from PMN [12] *ex vivo*. Our study extends these observations and confirms that intraperitoneal α1Pi during peritonitis is functionally deficient *in vivo*.

In conclusion, bacterial peritonitis in CAPD patients leads to intra-peritoneal elastase release from recruited PMN. The normal inhibitory processes designed to limit elastase-mediated tissue damage are locally inefficient, resulting in free elastase activity in nM concentrations. We suggest that this enzyme activity may have important implications for the pathogenesis of peritoneal membrane damage and the propagation of the inflammatory response during peritonitis.

Acknowledgments

KD is supported by a grant from the Welsh Scheme for the Development of Health and Social Science (WDSR J1143W36C). This work has been presented in part at the UK Renal Association, March 1992. We would like to thank Dr. Richard Fifield and the protein reference laboratory Cardiff Royal Infirmary for the α1Pi assay.

Reprint requests to Dr. K.L. Donovan, Institute of Nephrology, Royal Infirmary, Cardiff CF2 1SZ, Wales, United Kingdom.

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